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14. ABSTRACT

Ovarian cancer spreads by exfoliation or migration. Cell migration is typical of embryonic cells and a few other kinds of normal adult cells, but inappropriate migration is usually suppressed. Consequently, cancer cells must "learn" how to migrate. One obstacle to migration is the hostility of the microenvironment of the target tissue to epithelial cells of another tissue. Once a cancer cell has migrated to a novel tissue site, it must learn to survive at that site, and part of that process involves modification of the local microenvironment. In this project, we are training ovarian cancer cells to live when in contact with cells from other organs, such as the liver or lung. The goal is to then explore differential gene expression that explains the adaptation of the ovarian cancer cells to the novel environment. We have devised an Intravital Video Microscopy approach to this problem in which MOVCAR cells labeled with green fluorescent protein are embedded in pseudo-organ tissue from a "tomato" mouse in a small microscopy chamber on dorsal skin fold of a mouse. One problem we encountered was that ovary tissue implanted in the chamber continued to ovulate, and this compromised the clarity of the chamber for microscopy. This was solved by titrating progestin to a high enough dose to prevent ovulation but a low enough dose to allow for MOVCAR tumor growth. We have also found that adaptive changes in gene expression that allow the MOVCAR cells to grow on pseudo-organ tissue persist when the cells are briefly cultured, and we have exploited this for initial gene expression analysis. We are proceeding with high-throughput microdissection and gene expression analysis to study the adaptive gene expression response. This work is important because this model comprises an unusually tractable model for metastasis in which cancer cell gene expression responses to the novel environment can be correlated with real time growth parameters.

15. SUBJECT TERMS
Ovarian cancer, gene expression, metastasis, intravital video microscopy

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INTRODUCTION

Ovarian cancer spreads by exfoliation within the peritoneal cavity or by physically crawling to surrounding tissues. Both are major modes of movement. This task of spreading, like other forms of metastasis, requires that the tumor cells acquire new phenotypic capabilities, such as the ability to attract and interact with vasculature, the ability to support themselves via autocrine functions when the paracrine support is not available, the abilities to grow and divide in the new environment, and presumably the ability to spread further one new tissue microenvironment to other new tissue microenvironments. These capabilities are acquired through mutations, including point mutations, indels, gene amplification, and chromosome-scale loss and rearrangement, and through epigenetic alterations that alter gene activity. It has been extremely difficult to study the acquisition of these new capabilities with respect to real time, mainly because in human disease, the important events cannot be anticipated and observed in real time. We have developed a pseudo-organ model for cancer growth in which minced organ tissue is placed in a dorsal skin-fold chamber for Intravital Video Microscopy (IVM). The minced pseudo-organ tissue revascularizes and recapitulates some of the stromal characteristics of the original organ. This tissue can then serve as a bed upon which tumor cell spheroids can be placed to mimic several aspects of cancer. There are advantages to this system that we discussed in our application and in our previous progress report, mainly with respect to being able to follow revascularization and detailed aspects of tumor growth, such as necrosis, apoptosis, and mitotic index.. In particular, we are able to determine when implanted tumor spheroids stop dying and begin to adapt to the new microenvironment of the pseudo-organ. Our main assumption is that pseudo-organs can be treated as sites of metastatic cancer. The over-arching goal of this project is to gain a better understanding of differential gene expression in ovarian cancer, in both the tumor and the stromal part of a tumor. Part of this was motivated by our understanding that tumor cells appear to evolve to not only alter their own gene expression, through mutation and epigenetics, but also to influence the surrounding microenvironment. This subject of microenvironmental manipulation by the tumor has become an important topic in cancer research, and we may have gained insights from an unanticipated angle that may bear on ovarian cancer metastasis. As a means to this end, we first needed to establish a pseudo-ovary model, and during work on this aspect of the project, we encountered two unexpected obstacles, both of which we have overcome. Although these obstacles have delayed the time course of our experiments, we have made substantial progress on a very difficult problem, and are well-positioned to achieve most of the original approved goals of the project. As a part of a similar project, we have introduced a simple, direct, and cost-efficient approach that we think should be included in this project.

BODY In our previous progress report, we described changes to the SOW that accomodated an important obstacle, namely, that H2b-Cherry is apparently toxic to MOVCAR cells. Without H2b-Cherry-transformed MOVCAR cells, we were essentially unable to proceed, and therefore devised an alternative strategy in which we successfully transformed MOVCAR cells with H2b-GFP, and were able to grow these H2b-GFP cells on pseudo-organ tissues from (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J)^{nude} mice, which fluoresce in the TRITC part of the spectrum (i.e. red). So, by adapting our cell line and mouse model, we were able to grow the necessary tumors, as of the last progress report. The following is the modified SOW that we proposed in the previous progress report.

TASK 1

1. Development of H2b-GFP-MOVCAR cells
2. Development of a H2b-GFP-MOVCAR/Tomato pseudo-organ model for ovarian cancer (Completed)

TASK 2

1. Intravital microscopy studies implanting different stroma, i.e., ovary, skin, lung and liver with and without H2b-GFP-MOVCAR cells using the Tomato pseudo-organ model
2. Transverse microtome to isolate tumor and stroma.
3. cDNA preparation.
4. Microarrays and data analysis (on stroma and tumor)
5. Start breading (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J)^{nude} mice (contingent upon failure of T-cell filtration to avoid host rejection).

6. Begin in situ hybridization and immunohistochemistry.

(12 months)

TASK3

1. Bone marrow transplantation of bone marrow from (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J)^{nude} mice to nude mice.
2. Real-time RT-PCR analysis of genes in bone marrow derived cells using different stroma
3. LCM capture of bone marrow-derived cells
4. cDNA preparation.
5. Microarrays and data analysis (on cells from the host immune system).
6. Complete in situ hybridization and immunohistochemistry

(12 months)

Figure 1 is an image of such tumors, which we presented previously and repeat here to make the point that, at that time, we had only succeeded in growing H2b-GFP MOVCAR tumors for a week (i.e. until D7). Shortly thereafter, the ovarian tissue in the chamber began to hemorrhage as it continued to ovulate. When this happens, the tissue becomes obscured by blood in the chamber, and the model is ruined. Each of these experiments, i.e. implantation of the pseudo-organ followed by implantation of tumor cell spheroids, takes about a month to develop, and on a sporadic basis, chambers would last for more than two weeks after implantation of the tumor spheroids, only to, once again succumb to hemorrhaging. We did not know, at first, that the hemorrhaging we were observing was a natural phenomenon associated with continued ovulation, rather, we suspected some hithertoo unobserved experimental artifact having to do with the surgical procedure, possibly infection. The sporadic, albeit frequent, nature of the phenomenon seemed to support this interpretation. However, observation of pre-ovulation phenomena suggested that ovulation might be the cause, and we explained the sporadic nature in terms of the physical portion of the ovary used as pseudo-ovary. The solution that suggested itself was to suppress ovulation using progestin. We tried this, and were able to maintain the chamber much longer, proving that ovulation was, indeed, the cause of hemorrhaging. However, we then observed, to our great concern, that the H2b-GFP MOVCAR tumors would survive but not grow during progestin treatment. It is known that ovarian cancer is less likely in women who use birth control pills containing progestin, and this raised the possibility that the progestin treatment needed to maintain the system might undermine the model by essentially preventing tumor growth. We now know, however, that tumor growth is dependent on the dose of progestin. Initially, we tried 30 mg/kg body weight of progestin, which prevents both ovulation and tumor growth; we were able to lower the level of progestin (which is administered orally) to 13 mg/kg body weight of progestin which prevents ovulation, but does not prevent tumor growth. In **Figure 2**, we show a growth curve, which shows the usual drop off due to cell death, and then a post-adaptation increase in tumor mass.

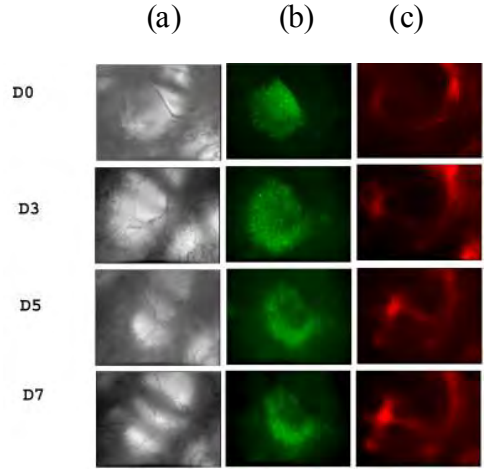

Figure 1. (a) Bright field, vasculature, (b) GFP-Movcar, (c) Tomato pseudo-ovary. Day 0-7 are indicated.

In this project, we plan to examine gene expression in the experimental tumors and stroma using microdissection, and we will discuss our progress on that specific aspect of the project later in this report. Before we proceed with that, however, we will describe another experimental strategy that we developed in a parallel funded project that addressed similar objectives in breast cancer, and which we propose to apply in this project, as well, because it is promising, inexpensive, and by its nature, is more robust than methods based on microdissection and purification from small tissue sources.

As we have discussed, the adaptation of cultured tumor cells to grow on pseudo-organ tissue requires a period of adaptation. This adaptation is important because it involves the genetic and epigenetic alterations that must take place for a cancer cell to be able to grow in the foreign environment of the metastatic site: by identifying the changes in gene expression, the essential adaptations are revealed. In turn, the genes behind

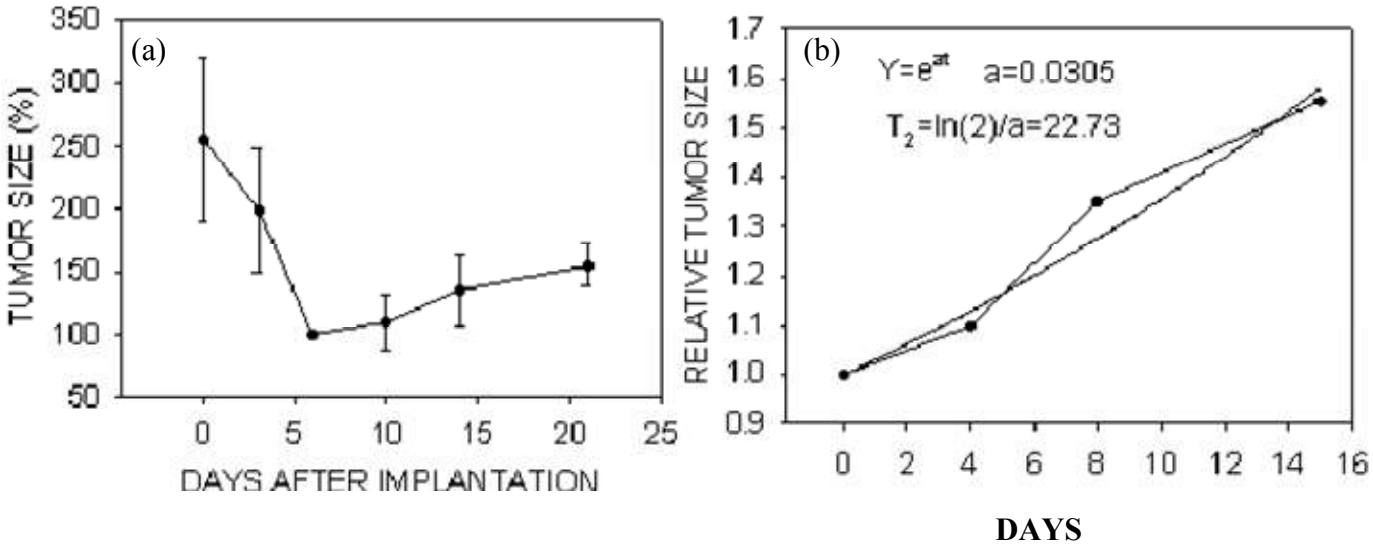

Figure 2. (a) Growth curve for MOVCAR tumor, normalized to Day 6. The initial decline, followed by recovery can be seen. (b) Calculation of growth rate from Day 6 and beyond for first training cycle.

these essential adaptations become potential targets for therapeutics to block metastasis. Adaptation in our system requires several passages of the tumor cells from animal to animal, and this entails intervening in vitro culturing of the cells to make transplantable spheroids. Invariably, each subsequent passage grows faster on the pseudo-organ, with much less initial cell death. This means that the cells growing in culture retain genetic and epigenetic features that allow them to grow on the pseudo-organ with shorter lag times, and this meant to us that gene expression analysis on the cultured cells might be worth doing. The benefits of working with cultured cells include that a single cell population can be used without significant contamination from other cell lines, which is a condition that is extremely hard to avoid when using microdissected tissue, and significant mass, which avoids needing amplification steps that can, in fact, always do, introduce error. The disadvantage is, of course, that the "snap shot" of gene expression is less relevant to the in vivo environment. Note, however, that candidates from the cell culture experiment can be tested very reliably by going back to the tissue, and that a work plan going in this direction is far simpler and more robust than direct analysis of tumor tissue.

We expanded cells from the first passage (Chamber model 1 or CM1) of an H2b-GFP Movcar tumor, and did gene expression analysis using Affymetrix microarrays comparing unadapted (CM)) to the CM1 adapted cells. The strength of this analysis derives from comparison with gene expression analysis in a parallel experiment involving the breast cancer cell line N202. That experiment is farther along than the ovarian project because we did not have the hemorrhaging problem. In **Figure 3,** we show, in the N202 experiment in which N202 cells are growing on pseudo-lung, Tnf, which is the mouse homolog of human TNF-alpha, is highly implicated in tumor cell adaptation. Not shown are similar results implicating Tnf when N202 cells grow on bone marrow. In **Fig. 3b**, however, MOVCAR cells growing on ovary do not adapt by a mechanism that involves overexpression of Tnf. Kim et al. [1] showed that Lewis lung carcinoma cells activate macrophage by secreting the proteoglycan versican, and the macrophage, in turn, secrete TNF-alpha and IL6. The mortality of $Tnf^{(-/-)}$ mice upon tail vein injection of LLC cells was markedly reduced, implying that Tnf is required for metastasis, and biological arguments suggest that Tnf exerts its role by conditioning the metastatic microenvironment. Here, we apparently have a cell line that has learned to secret Tnf without the macrophage intermediate. MOVCAR cells *do not* use this mechanism, and we hypothesize that this is because, in this experiment, the MOVCAR cells are growing on their native tissue of origin (i.e. ovary), whereas the N202 cells have been forced to adapt to what is essentially a metastatic environment. In other words, the N202 cells recognize the metastatic environment as "foreign" and have adapted via enhancement of factors normally associated with innate immunity. The suggestion, then, is that if we force MOVCAR cells to adapt to a tissue other than ovary, that we may also see adaptation involving Tnf. It is also possible, however, that MOVCAR cells adapted to tissue of a metastatic site do so indirectly, in which case, we can use the conditioned medium

strategy of Kim et al. [1] to track this down. Growth of MOVCAR on other tissues remains part of the experimental plan, and we are proceeding with these experiments.

Here, we briefly summarize a few of the more obviously interesting genes in our first run of this experiment on the adapted MOVCAR tumors. Genes were sorted according to the most extremely upregulated upon adaptation. The most extremely differentially expressed gene after adaptation was **Slfn3** (26-fold over-expressed), and very little is known about it. Second on the list, however, is **Il2rg** (interleukin 2 receptor gamma), which is *19-fold* overexpressed in CM1 relative to CM0. Il2rb is 4-fold higher, whereas Il2ra is not altered (0.9 fold). Il2rg is the common gamma chain important in signalling from a number of interleukins, including 2, 4, 7 and 21, and activates JAK3, and therefore the JAK-STAT pathway. Constitutive activation of JAK3 is observed in colorectal cancer, and repression of JAK3 leads to apoptosis [2]. JAK3 is constitutively active in acute lymphoblastic B-cell leukemia [3], and is considered a potentially viable target for drug development for leukemia. It is also upregulated, along with Il2ra and Il2rb in infiltrative human breast cancer[4]. *Our result is consistant with the possibility that gene amplification of Il2rg may constitutively activate JAK3, and if it generalizes to human ovarian cancer, suggests that such kinase inhibitor development may also apply to ovarian cancer*. IL2RG has been shown to be highly expressed in 3 out 12 ovarian adenocarcinomas; therefore, we may have devised a model for this type of ovarian cancer. The next logical step will be to determine whether this gene is amplified at the DNA level. However, before we embark on gene-by-gene analyses, we will want to extend these experiments to later steps in adaptation.

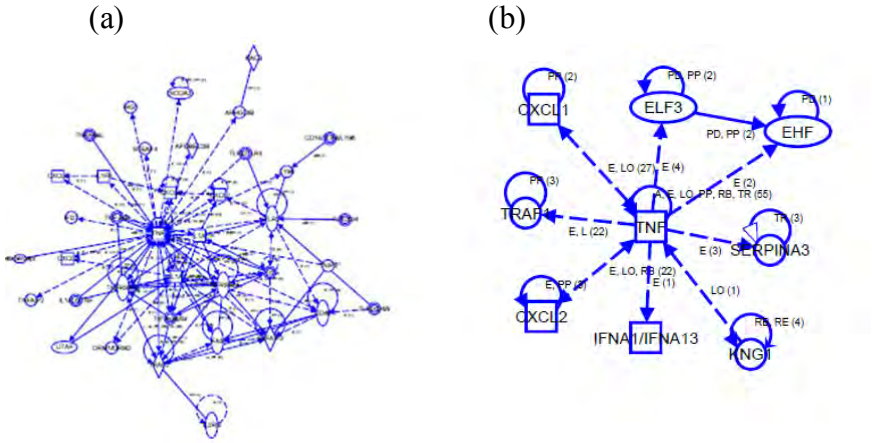

Figure 3. (a) Connectivity analysis of the top 40 adaptive genes in N202 breast tumor cells growing on lung tissue. Tnf is among the top 40 most highly differentially expressed genes between trained and untrained cells, and this connectivity diagram shows that the Tnf pathway is strongly implicated in adaptation. (b) Analysis of the connectivity of Tnf to adaptive genes in MOVCAR mouse ovarian tumor cells growing on pseudo-ovary tissue. Tnf does not become differentially expressed in this system, nor do other genes in the TNF pathway, as demonstrated by the low and indirect connectivity in this diagram.

These CM1 cells have gone quite far in avoiding apoptosis. **Nlrp1b** is a pro-apoptotic gene that forms part of the caspase-1-activating complex known as the inflammasome. In MOVCAR cells that have adapted for growth on pseudo-ovary tissue, Nlrp1b is over-expressed relative to the unadapted cell line by a factor of ~8. NLR proteins are thought to be "pattern matching" proteins that detect infectious agents as part of the inate immune system, and may also respond to stress; Nlrp1b is known to mediate cell death in response to anthrax toxin. The pro-apoptotic activity of Nlrp1b is opposed by Bcl-2 and Bcl-XL (Bcl2l1), which bind to Nlrp1b, and prevent its recruitment of caspase-1. In MOVCAR, 11 out of 19 NLR family members are over-expressed by at least a factor of 2, the highest being Nlrp1b at 8-fold, and the second being Nlrp3 at 4.5-fold. Interestingly, many Bcl-2 family members (8/14) are also over-expressed by at least a factor of 2, suggesting that an aspect of the growth of these ovarian cancer cells in pseudo-ovary tissue may be that the Bcl-2 family is opposing natural tumor suppressor activities of Nlrp1b. Importantly, we think, Il2rg + Il2rb, both of which are overexpressed, induce Bcl-2 and Bcl2l1 expression, and, indeed, these are over-expressed 2.6-fold and 2-fold, respectively. It is as if there is a battle raging with Nlrp1b trying to induce apoptosis while Il2rg is opposing apoptosis and winning. Interestingly, in comparison with GEO, human NLRP1 is highly expressed in ovarian cancer and in ovarian endometriosis.

Other genes are intriguing. **Cxcl1**, otherwise known in humans as GROα, is >11-fold over-expressed in ovary-adapted MOVCAR cells. This is interesting because Cxcl1 has been shown to induce proliferation in epithelial ovarian cancer cells by transactivation of the EGFR [5]; it is overexpressed in gastric cancer[6]; it promotes angiogenesis and invasion in gastric cancer [7]; and may mediate tumor invasion in bladder cancer[8]. CA125 is only mildly up-regulated (1.4-fold), but other mucins are highly upregulated (Muc19, 5-fold). March8 is 3-fold up-regulated, hnrnpa0 is 1.7-fold up-regulated, and others identified in microarray analysis of human tumors. Myc is 2-fold up.

We propose to repeat these gene expression analyses for successive adaptive cycles. The cost to the project is relatively small, yet the potential for insight is substantial. We are particularly interested in whether components of the innate immune system will be up-regulated when H2b-GFP MOVCAR cells are grown on pseudo-organs other than the ovary, and we are interested in whether we see the same adaptive trends upon repetition of the experiment.

The microdissection aspect of the project has, of course, been delayed by the problems we encountered, first, with the toxic mCherry, and second, with the ovulation-dependent hemorrhaging. Both of these problems have been solved, and we are beginning to make progress with microdissection. We have started using a Pannomics fluorescent scanning microscope (made by 3DHistotec) for the fluorescence microscopy stage of the experiment. The platform has limitations with respect to focusing on different fields in which the signal intensity may be low. We are resisting the temptation to use DAPI because of its nucleic acid binding capabilities, and we want to avoid energy transfer to the RNA (which may damage it), but its specificity for double stranded nucleic acid may make it workable. Regardless, the images are of sufficient quality that we can proceed. **Figure 4** shows an image of a thick section (16 microns thick). The thick section is in preparation for

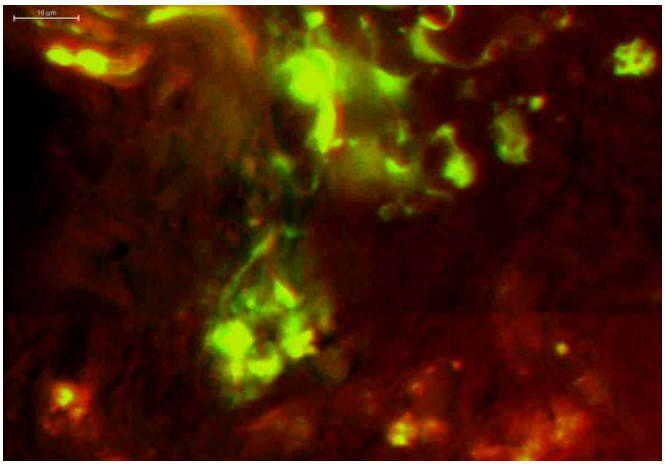

Figure 4. 16-micron thick sections of MOVCAR tumors growing on pseudo-ovary. The MOVCAR cells fluoresce green on a red "tomato" background. Scale bar = 10 microns.

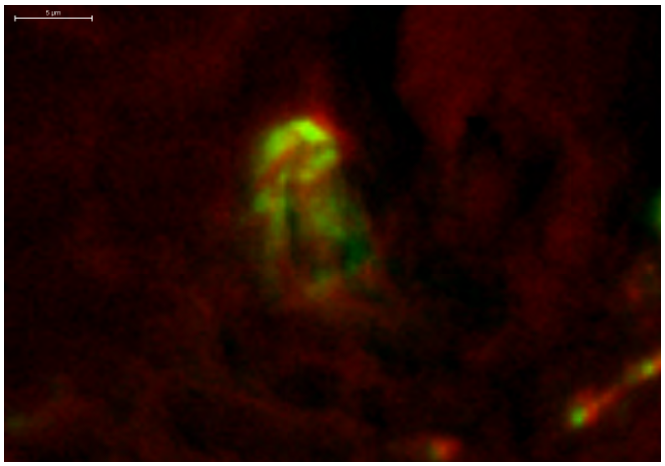

Figure 5. Higher power image of an H2b-GFP MOVCAR cell partially obscured by red protrusions, possibly vessels, from the revascularized ovary tissue. Scale bar = 5 microns.

microdissection of red (pseudo-ovary) and green (MOVCAR cells) regions. In this view, MOVCAR tumor cells are obvious. **Figure 5** shows a high power image of an H2b-GFP MOVCAR cell (or cells) partially obscured by red extensions that are probably microvessels. We have microtiled this same section.

We have developed a rapid microdissection platform that can render a section of tissue into thousands of small tiles (e.g. 150 microns sq.) in a matter of hours. The tumor represented in Figures 4 and 5 has been so dissected. The idea, here, is first that microdissection of tumor tissue always entails contamination with normal or stromal components or host immune cells unless one focuses on microdissection of single cells. However, the single cell approach is extraordinarily difficult and error prone, especially for down-stream RNA work. An alternative is to use larger microdissected piecs and a mixture model, with fluorescence or another marker, such as SNPs, to estimate the contributions of the various components. While single cell dissection has been possible, there are only a few publications on the subject because it is so difficult, whereas the mixture model

approach is far more robust, e.g. Stuart et al. [9]. **Figure 6** shows just one field of the tumor in Fig. 4 and 5 that has been microdissected into 150 micron sq. tiles, which now reside in 9 384-well plates. The blue grid shows the position of each tile, and the red box shows several tiles in this subset of the image that are enriched for

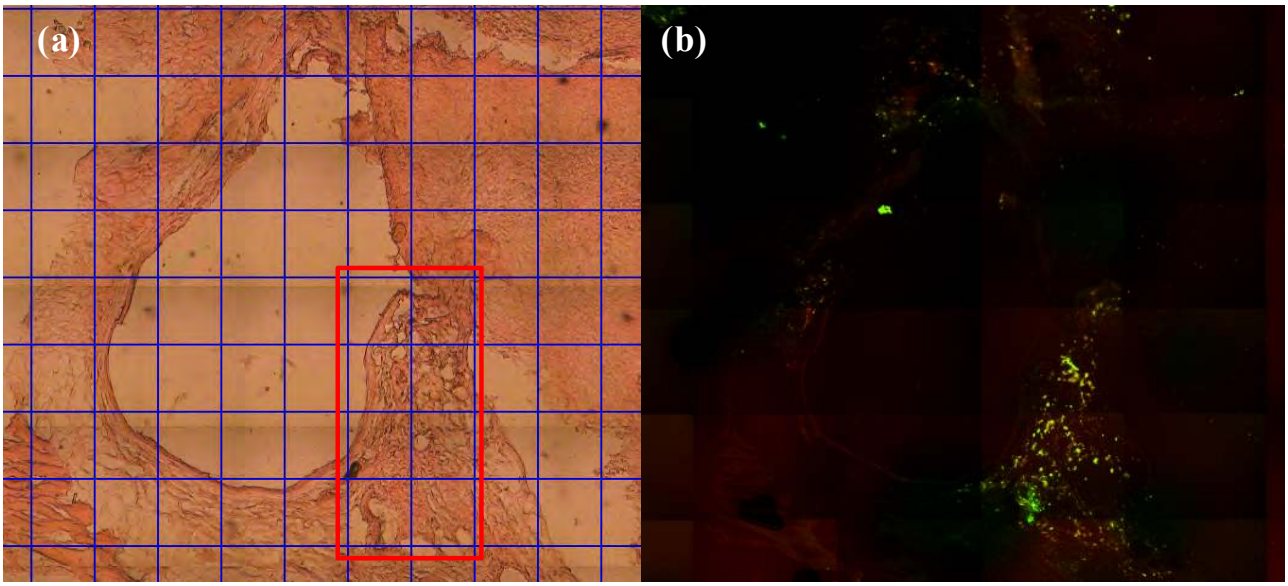

Figure 6. (a) Grid showing tiling pattern in a section of tumor CM2. The red box is enriched in H2b-GFP MOVCAR tumor cells, shown in (b).

H2b-GFP MOVCAR tumor cells. Our goal is to pool such tiles taken from this and additional sections from CM2 and perform gene expression profiling comparing such tumor-enriched tiles and tumor-poor tiles, as per our SOW. We will also do this with other stages, including CM1, 3 and 4, and we will do it for tumors trained to tissues other than ovary, also as per our SOW.

The main goal of this proposal is to study gene expression changes that accompany the adaptation of cancer cells to a novel environment that mimics the various metastatic environments to which ovarian cancer spreads. This is a two-step process, the first step being establishment of the IVM pseudo-organ model and the second being gene expression analysis on microdissected tissue. We anticipated that setting up the model would be straightforware, and it was not. Clearly, due to the obstacles we encountered with respect to the toxicity of the mCherry and subsequently the ovulation-dependent hemorrhaging, we have had to deviate from the approved statement of work. These problems are now solved, and we are proceeding with the further substantive aspects of the proposed research, though in a modified and somewhat compressed time-frame. To date, the model is established, we have two of the anticipated 4 training cycles for pseudo-ovary completed, and we have mice in which lung and liver tissue are revascularized in anticipation of implantation, we have, in parallel, the gene expression analysis strategy based on expansion of the trained cells in culture. Below is a proposed modification to our SOW in which we summarize our progress and indicate our proposed activities for the final year of this award.

TASK 1

1. Development of H2b-GFP-MOVCAR cells
2. Development of a H2b-GFP-MOVCAR/Tomato pseudo-organ model for ovarian cancer

Completed by the end of the first year.

TASK 2

1. Solving the ovulation/hemoraging problem with progestin. **Completed in the second year**.
2. Intravital microscopy studies implanting different stroma, i.e., ovary, skin, lung and liver with and without H2b-GFP-MOVCAR cells using the Tomato pseudo-organ model. **The first and second training cycles on ovary are now complete. We have prepared the first sets of animals with lung and liver, which will be ready for implantation of spheroids from CM2.**

3. Microdissection to isolate tumor and stroma. **We have microdissected tumor tissue from CM1, and have CM2 tumors ready to microdissect.**
4. Gene expression on sorted, cultured CM1. **Completed, but we propose to repeat this to achieve stronger statistics.**

TASK3

1. ***We propose*** to continue with the gene expression in sorted, cultured adapted cells as this appears to be revealing some genes that are already known to be differentially regulated in ovarian cancer.
2. Microarrays and data analysis on stroma and tumor. **This will be the main task of the final period.**
3. Our original plan was to breed (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J)^{nude} mice for Aim 2, in which we isolate cells of the immune system to subject to microarray analysis. This breeding is not necessary, because we have learned to use T-cell filtration during bone marrow transplantation. **We will irradiate nude mice, transplant T-cell filtered bone marrow from Tomato mice, and isolate immune system cells from the tumors.**
4. In situ hybridization and immunohistochemistry in human tissues. **This will be done on a more limited basis, given that the appropriate data will be generated in this final year. However, we will do a good job on this from the bioinformatics perspective.**

Realistically, with the time remaining in this grant, we expect to be able to do an excellent job on gene expression in the various pseudo-organ experiments, and an excellent job on gene expression in the cultured, trained cells. We expect to at least be able to do exploratory-level experiments on immune system components. Aim 3, which relates to validation in human tissues can be achieved, perhaps less thoroughly, although the bioinformatic infrastructure for ovarian cancer has improved somewhat, over the past two years, and we can also use realtime PCR. Of course, we will do a thorough a job as possible with the available resources, but given the difficulties we encountered with the model early in the project, we think it makes more sense to concentrate on those aspects of the project that are working, in order that we make a significant contribution vis-à-vis adaptation, rather than superficial contributions in a broader range of subjects (i.e. adaptation of the immune system).

KEY RESEARCH ACCOMPLISHMENTS

- Development of a H2b-GFP-MOVCAR cell line
- Development of a H2b-GFP-MOVCAR/Tomato pseudo-organ model for ovarian cancer: specifically, we have overcome the mCherry problem and we have overcome the ovulation-dependent hemorrhaging problem.
- Gene expression analysis after the first training cycle.

REPORTABLE OUTCOMES

- The H2b-GFP-MOVCAR cell line deserves to be made publically available, as it is likely to be useful in ovarian cancer research using diverse strategies.
- The H2b-GFP-MOVCAR/Tomato pseudo-organ ovarian cancer model is reportable without much additional refinement, and we are preparing a manuscript. We need a few high-quality FFPE images.
- Our gene expression analysis shows that cells retain a pattern of differential gene expression from training even after they have been expanded briefly in culture. We will report this in a publication, but we first want gene expression analysis in other pseudo-organs to test the hypothesis that the cancer cells autonomously activate components of the innate immune system when they find themselves in the wrong tissue environment.

CONCLUSION Prior to this project, we had devised pseudo-organ models for lung, liver, brain, bone marrow, breast, and prostate and anticipated that we would encounter no significant problems with essentially

any other tissue. The toxicity of mCherry to MOVCAR cells aside, this assumption was incorrect; it simply had not occurred to us that ovary would present its own set of unique problems. Not only did ovulation-dependent hemorrhaging present a problem, but control of that through hormone therapy presented its own set of problems vis-à-vis the effect of progestin on ovarian tumor growth. It was fortunate that a dose of progestin could be found that both suppressed ovulation and permitted tumor growth, for without this result, IVM would not be possible on pseudo-ovaries. We will now proceed with our gene expression studies.

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